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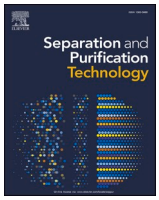

Extraction and purification of short DNA fragments from plasma using aqueous two-phase systems for liquid biopsy

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ABSTRACT

Liquid biopsy is a minimally invasive method for tumor profiling, relying on detecting and analyzing tumor-derived biomarkers present in bodily fluids. Key biomarkers, such as circulating cell-free DNA (cfDNA), are often present at very low concentrations in plasma. Efficient extraction and purification of cfDNA are critical, as impurities such as proteins and salts can interfere with downstream applications like next-generation sequencing. This study demonstrates the potential of aqueous two-phase systems (ATPS) as a capture step for short DNA fragments from human plasma, building on prior findings with synthetic systems. DNA partitioning, recovery, and separation from plasma proteins were evaluated in selected polyethylene glycol (PEG)/phosphate/plasma ATPS. PEG 1000 systems proved to be more effective than PEG 400 systems, achieving up to 90 % DNA recovery in the bottom phase. Total protein concentration was reduced to 3 mg/ml, while most of the protein was removed as reversible aggregates forming an interphase. System robustness was confirmed using plasma from various donors and blood collection tube types, showing consistent DNA recovery and phase separation behavior. To address dilution caused by liquid phase-forming components, a modified ATPS design using solid PEG and phosphate was implemented. This approach increased plasma input from 37.7 % (w/w) to 66.7 % (w/w) without altering DNA or protein partitioning, effectively maximizing the plasma load for extraction. Lastly, a reverse elution protocol using purification plates containing a desalting matrix was introduced to concentrate the DNA-containing phase while removing salts and residual proteins, improving its suitability for downstream sequencing-based applications.

1. Introduction

Cancer remains one of the leading causes of death worldwide, with the global cancer burden projected to rise by 77 % by 2050, according to the International Agency for Research on Cancer [1]. This underscores the need for precise genetic profiling to improve cancer diagnosis and treatment. Tissue biopsy, the gold standard for tumor characterization, is limited by its invasive nature and dependence on single, localized tumor samples.

Liquid biopsy has emerged as a minimally invasive alternative to overcome these limitations, particularly for tumors inaccessible through surgery [2]. Liquid biopsy analyzes cancer-derived biomarkers found in the bloodstream and other bodily fluids, enabling tumor profiling from a simple blood draw. Its ease of repetition and ability to capture tumor heterogeneity make liquid biopsy a versatile tool for various applications across disease progression, including early cancer detection, monitoring treatment response, tracking residual disease post-surgery, and identifying emerging tumor clones [3].

Among the various biomarkers accessible through liquid biopsy, circulating cell-free DNA (cfDNA) holds particular promise. This double-stranded DNA is released primarily during apoptosis, resulting in highly fragmented DNA with a predominant length of 167 bp [4]. A small fraction of cfDNA originates from tumor cells as circulating tumor DNA (ctDNA), which is often even shorter, typically ranging from 132 to 143 bp [5].

The cfDNA-based liquid biopsy workflow typically begins with blood

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collection in specialized stabilizing tubes to prevent contamination by genomic DNA (gDNA) from lysed blood cells [6]. Plasma is then fractionated using a double centrifugation protocol to maximize recovery and remove residual blood cells [7]. cfDNA is extracted from the plasma fraction, purified, and concentrated for sequencing-based analysis. Techniques such as digital droplet PCR (ddPCR) or next-generation sequencing (NGS) are employed based on the required sensitivity and coverage [8]. Although sequencing yields actionable insights into tumor genetics, cfDNA extraction remains a critical bottleneck, requiring high recovery and purity from minimal starting material, typically only 1–40 ng/ml of plasma [9]. Commercial cfDNA extraction kits primarily rely on DNA adsorption to solid phases, such as silica membranes or magnetic beads, in the presence of chaotropic salts [10]. While effective, these methods can lead to residual chaotropic salt contamination, which inhibits amplification-based assays. To address this, standard kits use bind-wash-elute protocols with multiple wash steps. However, this can reduce overall recovery and introduce a fragment length bias due to uneven extraction efficiency, particularly of short fragments [1112]. These limitations highlight the need for novel approaches to cfDNA extraction that improve efficiency, scalability, and standardization, facilitating the broader implementation of liquid biopsy analyses in clinical settings.

Aqueous two-phase systems (ATPSs) provide a promising, low-cost, and rapid alternative for initial DNA capture and concentration, particularly in biochemical and analytical diagnostic applications such as liquid biopsy, where scalability and automation are critical [13]. By adding phase-forming components, such as polymers and salts, directly to plasma, ATPS can enable the selective partitioning of cfDNA into one phase while enriching other plasma components, such as proteins, in the opposite phase. To ensure compatibility with downstream analysis, an additional purification step is required to remove residual phase-forming components and contaminants that may interfere with sensitive detection techniques. For DNA collected in the polyethylene glycol (PEG)-rich top phase, alcohol-induced precipitation is a viable approach [14]. When DNA is found in the salt-rich bottom phase, desalting resins or reverse elution chromatography can be utilized. These methods allow the purified DNA to be recovered in the flow-through, while salt and other impurities are effectively retained [15].

Studies on ATPS-based DNA extraction have predominantly focused on gDNA [1617] or plasmid DNA (pDNA) [1819], which differ significantly from cfDNA in both size and conformation. Detailed insights into the partitioning behavior of short DNA fragments that mimic cfDNA [14] remain limited. Additionally, most existing studies investigate partitioning effects in clean ATPS or through single-solute experiments [20]. However, it has been reported that biomass in ATPS can influence phase formation, often causing shifts in the binodal curve [21].

Available studies exploring ATPS loaded with whole blood [22] or plasma [23] typically focus on separating blood proteins, such as albumin, immunoglobulin G (IgG), or hemoglobin, with little or no mention of DNA. Selvakumar et al. [20] describe an approach to increase biomass loading by introducing solid phase-forming components, such as undissolved PEG and salts, instead of relying on pre-dissolved solutions. This allows the substitution of the liquid typically used to dissolve the phase-forming chemicals, such as water, with biological samples like plasma containing a high amount of water.

In a previous study, we investigated the partitioning behavior of a 160 bp DNA fragment in clean PEG/salt ATPS using automated routines on a liquid handling station [24]. Optimal conditions for short DNA fragment recovery were identified through high-throughput screening, and experiments with selected system points were carried out using a synthetic plasma solution. The results demonstrated that a PEG 1000/phosphate system led to selective DNA partitioning into the bottom phase, with proteins mainly remaining in the top phase. Furthermore, the addition of neutral salts, such as NaCl, to a PEG 400/phosphate system significantly shifted DNA partitioning from the top to the bottom phase without affecting protein distribution. Both systems achieved high DNA recoveries of around 87 % with substantial protein removal, meeting the criteria for selective cfDNA recovery. However, it remains uncertain whether the partitioning behavior and separation efficiency observed in synthetic systems can be replicated when using a complex biological matrix like human plasma in ATPS.

Based on the insights described above, we present data on the distribution of short DNA fragments in ATPS containing human plasma. The robustness of the proposed ATPS capture step is evaluated using blood collected from individual donors and stored in various tube types. To increase plasma input volume and minimize the dilution of target DNA by phase-forming components, an ATPS design incorporating solid PEG and salts is introduced. In addition, further purification and simultaneous concentration of the DNA-containing phase are explored using an adapted reverse elution protocol. This study establishes a foundation for developing an ATPS-based cfDNA extraction process tailored for clinical liquid biopsy applications.

2. Material and methods

2.1. Chemicals and stock solutions

PEG 400 (Sigma-Aldrich, St. Louis, MO, USA) and PEG 1000 (Merck KGaA, Darmstadt, Germany) were used in synthesis grade. $NaH_2PO_4{\cdot}H_2O$ and NaCl were obtained from Merck, while K_2HPO_4 was purchased from VWR International (Radnor, PA, USA). PEG and salt stock solutions, along with purified 160 bp DNA fragments for spiking experiments, were prepared as described by Meutelet et al. [24].

2.2. Acquisition and processing of blood samples

Blood samples were collected from healthy donors at the Institute of Experimental Hematology and Transfusion Medicine at the University Hospital Bonn (Bonn, Germany). The study procedures were performed in compliance with the approval granted by the Ethics Committee of the Medical Faculty at the University of Bonn (protocol code 070/05) and adhered to the Declaration of Helsinki. Written informed consent was obtained from all participants, and the procedures were carried out in accordance with institutional guidelines. After giving consent, approximately 10 ml of whole blood was collected into one of the following stabilizing blood collection tubes: EDTA, PAXgene® Blood ccfDNA Tube (PreAnalytiX GmbH, Hombrechtikom, Switzerland), or Cell-Free DNA BCT® (Streck, La Vista, NE, USA). Blood samples were shipped at room temperature and processed within 24 h using a double centrifugation protocol to maximize plasma recovery. Whole blood was centrifuged in the collection tubes at 1600 x g for 10 min at room temperature. The plasma layer was then collected and centrifuged again at 16000 x g for 10 min to remove any remaining blood cells. Plasma aliquots of 1 ml were stored at −80 °C until further use.

2.3. ATPS preparation and sampling

All ATPS partitioning experiments were performed at room temperature in an air-conditioned laboratory to minimize temperature-related variability in phase behavior. Systems were prepared with a physiological pH of 7.4 by adjusting the salt ratio in the phosphate buffer stock solution. A schematic overview of the experimental workflow is provided in Fig. S1 in the supplementary material, and includes the following main steps: ATPS preparation, sampling of the individual phases, sample dilution, and subsequent analysis. Key system characteristics, including binodal coefficients and tie-line slopes, are summarized in Table S2. The methods used for automated binodal and tie-line determination are described in detail in Meutelet et al. [24], along with the corresponding phase diagrams.

Plasma aliquots were thawed at room temperature on the lab bench for 45 min. Any sedimented material was left undisturbed to avoid resuspension. ATPS with a total volume of 600 µl were manually

prepared in 1.5 ml Eppendorf tubes by sequentially adding plasma, 160 bp DNA fragments (final concentration of 200 ng/ml per ATPS), 40 % (w/w) phosphate stock solution, and 70 % (w/w) PEG stock solution. The specific volumes of components used to prepare each system point are listed in Table S1. For each ATPS composition, a blank control was prepared by spiking plasma with water instead of DNA stock to allow background correction. After thorough mixing by vortexing, phase separation was accelerated by centrifugation at 8000 x g for 5 min. Phase sampling and volume determination were performed with care using a pipette. The top phase was first collected and transferred to a new tube without disturbing the interphase or bottom phase. The bottom phase was then recovered by gently pressing the interphase layer against the tube wall. Remaining liquid trapped in the aggregated interphase was removed by brief centrifugation before the interphase was resuspended in 100 µl water. All samples were then diluted tenfold in water to reduce interference of phase-forming components during quantification analyses.

2.4. ATPS preparation with solid components

To increase the plasma input volume without altering the amount of phase-forming components in the ATPS, PEG and salts were added directly to the plasma as solids rather than pre-dissolved stock solutions. Waxy PEG cylinders of the appropriate weight were prepared by melting PEG 1000, solid at room temperature, in a microwave and allowing it to harden in a cylindrical mold for a few minutes. The PEG cylinders and phosphate salts were then added to 1.5 ml Eppendorf tubes containing plasma spiked with 160 bp DNA fragments. To ensure complete solubilization, the tubes were placed on a thermo-shaker at 25 °C for 5 min, briefly vortexed, and mixed for an additional minute. After a final vortexing step, the tubes were centrifuged at 8000 x g for 5 min to achieve phase separation. Sampling and sample preparation for analysis followed the same protocol used for ATPS prepared with pre-dissolved components. The amount of phase-forming components and plasma used to prepare the investigated system points is specified in Table S3.

2.5. Combined DNA purification and concentration

Reverse elution plates from the EchoCLEAN Organic Solvent DNA CleanUp Kit (BioEcho, Cologne, Germany) were tested as a combined DNA purification and concentration step from the salt-rich phase. This reverse elution method uses a purification matrix that retains impurities and salts, allowing DNA fragments to be recovered in the flow-through. The plates were used according to the manufacturer's instructions with slight modifications to the commercially available product. Specifically, a custom matrix filling volume of 500 µl was used, and various centrifugation speeds were tested during conditioning and elution to optimize DNA recovery and concentration. Each well was loaded with 100 µl of a 200 ng/ml 160 bp DNA solution, and each combination of conditioning and elution speed was tested in triplicate. To assess the desalting capacity of the matrix, conductivity measurements were performed using the conductivity sensor of an ÄKTApurifier system (Cytiva, Uppsala, Sweden).

2.6. Analytical methods

2.6.1. DNA quantification

DNA was quantified using the Quant-iT™ PicoGreen™ dsDNA HS assay kit from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Black 384-well microplates with a total volume of 20 µl per well were used for the top, bottom, and interphase samples. Each sample was analyzed alongside blanks with similar composition but without DNA to account for any interference from phase-forming or plasma components with the assay. These blank phases were also spiked with a known amount of 160 bp DNA fragments to generate a reference signal for recovery calculations. Fluorescence measurements were conducted according to the manufacturer's instructions after an incubation period of 2 min. Each sample, blank, and reference was measured in triplicate. DNA recoveries in each phase were calculated by multiplying the measured DNA concentrations by the respective phase volumes and dividing by the amount of input DNA.

2.6.2. Protein quantification

Total protein concentrations in the ATPS phases were measured in triplicate using the NanoDrop™ 2000/2000c UV/Vis spectrometer (Thermo Fisher Scientific Inc.). Water was used as a blank, and plasma used in the ATPS was diluted tenfold and measured to serve as a reference for recovery calculations. An average extinction coefficient of 10 was assumed for the plasma protein absorbance measurements at 280 nm.

2.7. SDS-PAGE analysis

Protein content in the phases was visualized using reducing SDS-PAGE with 50 mM DTT. Phase samples were diluted tenfold in water or purified using reverse elution plates (BioEcho) before mixing with NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA, USA) and denaturation at 70 °C for 10 min. Proteins were separated on NuPAGE 4–12 % BisTris Mini Protein Gel using an Xcell SureLock Mini-Cell (Invitrogen) at 200 V, stained with InstantBlue Coomassie protein stain (US Biological, Salem, MA, USA), and de-stained in water. A Novex Mark12 protein standard (Invitrogen) was used as a molecular weight marker, and gels were imaged with a GelDoc Go system by Bio-Rad (Hercules, CA, USA).

3. Results and Discussion

3.1. DNA capture from plasma using ATPS

3.1.1. ATPS formation

The applicability of previous findings from synthetic plasma experiments was assessed for ATPS prepared with human plasma. In preliminary experiments, phase formation of an exemplary PEG/phosphate/plasma ATPS was investigated, as shown in Fig. 1. Upon adding PEG and salt solutions to plasma, initial turbidity indicated the formation of protein aggregates [25]. After centrifugation, the resulting ATPS exhibited three distinct regions: a yellowish top phase, a visibly defined interphase, and a clear bottom phase. These findings suggest a three-phase partitioning system with a solid interphase rather than the conventional aqueous two-phase partitioning typically observed in synthetic systems. However, it remains unclear whether DNA detected in the interphase actively partitions into this region or if it co-precipitates or becomes physically trapped, thereby altering its initial partitioning behavior. The thickness and appearance of the interphase varied with the specific ATPS composition. For example, PEG 1000 systems formed thicker and cloudier interphases compared to PEG 400 systems, where the interphase appeared as a thin, compact disk. This observation aligns with the higher precipitating power of PEG with increased molecular weight [26].

Similar to synthetic PEG/phosphate systems, the top phase of the plasma-containing ATPS was primarily composed of polymer, while the bottom phase consisted predominantly of phosphate salts. After removing all liquid constituting the top and bottom phases, the interphase was redissolved in water, indicating it consisted predominantly of reversibly aggregated proteins. This enabled the analysis of DNA and protein content in the interphase, facilitating the closure of mass balances which was previously impossible for clear, synthetic systems lacking a visible interphase.

Interestingly, aside from the formation of an interphase, the observed phase volume ratios were comparable to those measured in control systems using water as the matrix instead of plasma. This indicates that replacing water with plasma does not significantly alter the

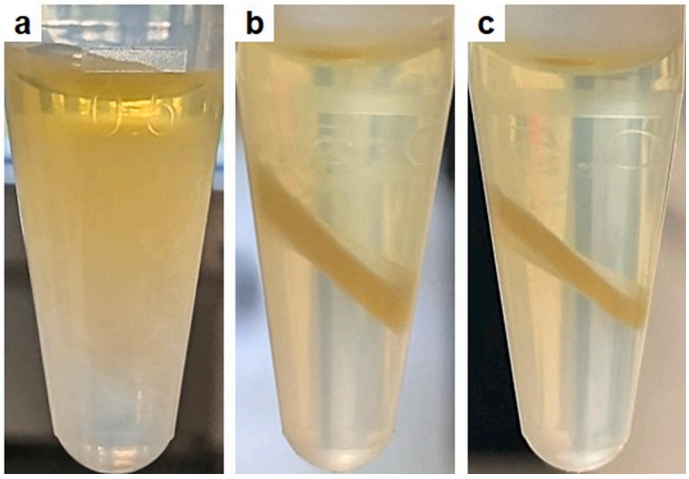

Fig. 1. PEG 1000/phosphate/plasma ATPS: (a) before phase separation, (b) after centrifugation at 2000 x g, (c) after centrifugation at 8000 x g.

phase equilibrium of the selected formulations, aligning with the findings of Selvakumar et al. for ATPS PEG 1000/phosphate ATPS loaded with bovine plasma [21]. Manually determined phase volumes for each system point are provided in Table S4.

Additional experiments revealed that centrifuging the mixed systems at a higher centrifugation speed of 8000 x g rather than the 1500 x g used for synthetic ATPS produced a slightly more compact and manageable interphase (Fig. 1b and c). This adjustment did not affect overall DNA and protein distribution but improved the removal of residual liquid from the interphase. Consequently, all plasma ATPS experiments were performed with phase separation at 8000 x g for better handling and consistency.

Although the DNA input concentration of 200 ng/ml used in this study exceeds typical cfDNA levels in plasma [9], it was chosen to ensure reliable detection with fluorescence-based assays. Since partitioning behavior in ATPS remains independent of concentration as long as solubility limits are not exceeded [27], the observed DNA distribution is expected to remain consistent at lower, clinically relevant DNA concentrations.

3.1.2. PEG 400/phosphate/plasma ATPS

In previous synthetic plasma experiments, DNA and protein were predominantly recovered in the top phase of a PEG 400/phosphate ATPS. However, the addition of NaCl resulted in DNA migrating almost entirely into the bottom phase, separating it from the main protein fraction. As explained in more detail in Meutelet et al. [24], this directed switch is mainly due to uneven ion distribution in the ATPS, which changes the physico-chemical properties of the phases [26]. The partitioning behavior of a 160 bp DNA fragment in a 17.7 % (w/w) PEG 400/17.3 % (w/w) phosphate ATPS containing 24.0 % (w/w) human plasma with and without NaCl was investigated for comparison. Fig. 2 shows the respective DNA and plasma protein recoveries in the top phase (TP), bottom phase (BP), and interphase (IP).

Unlike the synthetic plasma system, where DNA was fully recovered in the top phase, DNA was almost evenly distributed between the top phase and the interphase. Protein distribution, in contrast, closely mirrored prior findings, even though the total protein concentration in the plasma sample was approximately 6 times higher than the 10 mg/ml human serum albumin (HSA) and IgG mix spiked into the synthetic solution. Upon adding NaCl to the plasma-based ATPS, DNA did not transition to the bottom phase as observed in synthetic plasma but was instead primarily retained in the interphase. These results suggest that the interphase interferes with DNA partitioning and phase transition, likely because of the physical barrier formed by aggregated proteins and potential DNA-protein interactions [28].

Interestingly, while the formation of an interphase may appear disadvantageous, it presents a potential opportunity for selective DNA capture. In this PEG 400 system, the interphase contains only a fraction of the total protein and is virtually free of PEG and salt, reducing the risk of contaminants that could interfere with downstream analyses [29]. DNA concentration can be significantly increased by redissolving the interphase in a small volume of water or buffer, making this approach a promising initial capture and concentration step for cfDNA from plasma.

Additional experiments demonstrated that DNA collected in the interphase could be concentrated 25-fold, from an initial concentration of 200 ng/ml in plasma to over 5000 ng/ml, by redissolving the

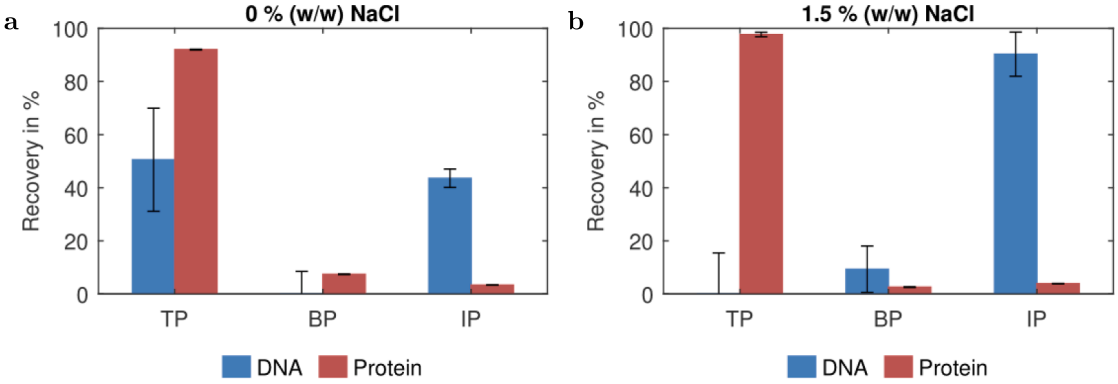

Fig. 2. 160 bp DNA and plasma protein recoveries in the top phase (TP), bottom phase (BP), and interphase (IP) of a 17.7 % (w/w) PEG 400/17.3 % (w/w) phosphate/24.0 % (w/w) plasma ATPS (a) without (b) and with 1.5 % (w/w) NaCl.

interphase in just 10 µl of water (data not shown). However, despite the low protein recovery in the redissolved interphase (3 % of the input amount), the resulting protein concentration was 21.3 mg/ml, far exceeding the tolerance thresholds for sequencing-based downstream analyses [30]. Performing a lysis step on the DNA-containing interphase, followed by an additional purification step, could be a viable option to reduce residual protein levels.

3.1.3. PEG 1000/phosphate/plasma ATPS

Building on the insights from earlier PEG 1000 ATPS investigations, where DNA partitioned into the bottom phase with minimal protein recovery, the distribution of DNA and protein in a 19.5 % (w/w) PEG 1000/13.8 % (w/w) phosphate/37.7 % (w/w) plasma system was analyzed (Fig. 3). For a detailed description of the observed partitioning behavior, see Meutelet et al. [24]. The higher molecular weight of PEG 1000 requires fewer phase-forming components compared to PEG 400 systems, allowing for a greater plasma input volume. This effect arises from the binodal shift towards the origin, attributed to the increased hydrophobicity and exclusion effects of higher molecular weight PEG [31]. Processing larger plasma volumes offers a distinct advantage, as the probability of detecting potential rare cfDNA variants present at very low concentrations is increased [3233].

With the ability to sample the redissolved interphase, we confirmed that the discrepancies in DNA and protein mass balances in clean systems without plasma were due to accumulation at the interface between the top and bottom phases. As expected from the synthetic plasma system experiments, up to 90 % of the spiked DNA was recovered in the bottom phase, while only 5 % of the total plasma protein, corresponding to a concentration of 3.1 mg/ml, was detected in the same phase. Reducing SDS-PAGE analysis (Fig. S2) was used to qualitatively assess the protein content in the phases of the PEG 1000 and PEG 400 systems. The results indicated that protein distribution varies with the polymer molecular weight and confirmed that protein recovery in the bottom phase is the lowest. The lower protein concentration left in the bottom phase than the interphase, along with the capability for higher plasma input, suggests that PEG 1000 systems are better suited than PEG 400 systems for efficient cfDNA extraction from plasma. This is why all following experiments were performed using PEG 1000 systems.

However, although the DNA concentration in the bottom phase can reach several hundred ng/ml, the protein concentration remains substantially higher. This poses a significant challenge for downstream sequencing techniques requiring polymerase chain reaction (PCR), as blood proteins like IgG, lactoferrin, and hemoglobin are known to be potent PCR inhibitors [34]. These findings highlight the inherent limitations of relying on an entirely ATPS-based system for extraction, as achieving sufficient DNA concentration and purity in a single step is not feasible. Nevertheless, as an initial capture step, ATPS proves to be an efficient and rapid method, removing over 95 % of plasma proteins while retaining high DNA recovery. Following this primary capture step, addressing the remaining challenges, namely removing salts and residual protein from the DNA-containing bottom phase, is critical for achieving the purity required for sensitive downstream analyses.

To the best of our knowledge, only one other study has described an ATPS-based extraction kit for cfDNA purification from plasma [14]. In that approach, cfDNA is first captured in the bottom phase of an initial ATPS, then transferred and concentrated in the top phase of a second ATPS, followed by isopropanol precipitation, washing, and redissolution. Other recent studies on nucleic acid extraction rely on different capture mechanisms, most commonly the adsorption of cfDNA onto silica matrices [35]. Examples include the use of silica-coated magnetic beads [3637] or column-based kits [3839]. A comprehensive overview of microfluidic systems for nucleic acid extraction from human samples is provided by Obino et al. [40]. While these methods generally report high cfDNA recovery and purity, ATPS-based extraction may offer distinct advantages in terms of simplicity, cost-effectiveness, speed, and scalability. A direct comparison of yield, purity, and overall handling will be performed once the extraction process incorporating the investigated ATPS as a capture step has been fully established.

3.2. System robustness

3.2.1. Blood collection tube variability

The performance of cfDNA analysis in liquid biopsy is affected not only by the low initial DNA concentrations and the challenge of sample purity but also by various preanalytical factors, including blood collection, processing, and storage conditions [41]. Several studies have investigated cfDNA quantity and integrity when stored in different types of blood collection tubes [4243]. While K2/K3EDTA-containing tubes remain the most commonly used devices, specialized blood collection tubes developed by companies like Streck or PreAnalytiX have been commercialized specifically for cfDNA storage [44]. These tubes contain anticoagulants and distinct stabilizing agents [6], which may impact DNA distribution and recovery in the ATPS capture step employed in this study. To evaluate the robustness of the selected ATPS against variability introduced by different blood collection tube types, the distribution of 160 bp DNA and plasma proteins was analyzed in 19.5 % (w/w) PEG 1000/13.8 % (w/w) phosphate/37.7 % (w/w) plasma systems. Pooled plasma samples from EDTA (E), Streck (S), or PAXgene (P) tubes were compared, as shown in Fig. 4.

Most DNA was consistently recovered in the bottom phase across all three tube types, with tubes S and E achieving similar recoveries over 75 %, while tube P recovered 66 %. As expected, DNA recovery in the top phase was minimal, with values below the detection limit for tubes S and E. Tube P showed the highest recovery in the interphase with 36 %, compared to tubes S (23 %) and E (17 %). However, the interphase measurements exhibited the highest standard deviations for all tube

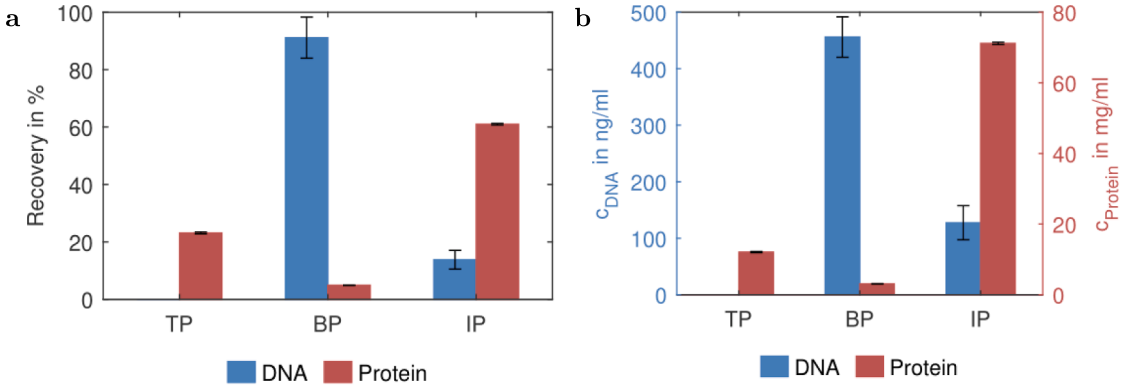

Fig. 3. 160 bp DNA and plasma protein (a) recoveries and (b) concentrations in the top phase (TP), bottom phase (BP), and interphase (IP) of a 19.5 % (w/w) PEG 1000/13.8 % (w/w) phosphate/37.7 % (w/w) plasma ATPS.

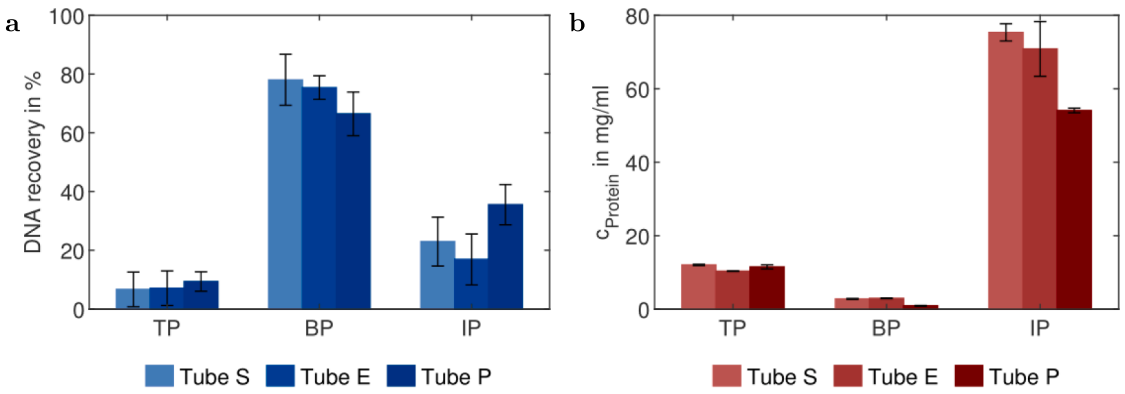

Fig. 4. (a) 160 bp DNA recovery and (b) plasma protein concentration in the top phase (TP), bottom phase (BP), and interphase (IP) of 19.5 % (w/w) PEG 1000/13.8 % (w/w) phosphate/37.7 % (w/w) plasma ATPS with pooled plasma from EDTA (E), Streck (S) or PAXgene (P) blood collection tubes. Partitioning experiments were performed in duplicate and samples were measured in triplicate.

types, likely due to protein interference that could have affected fluorescence readings [45]. Protein concentration measurements confirm that DNA capture in the bottom phase is a viable option, as protein concentration remains below 3 mg/ml for all tube types. The most significant difference was observed in the interphase, where protein accumulation was lowest for tube P (54 mg/ml) compared to tubes S and E (over 70 mg/ml).

Interestingly, tube P exhibited higher DNA recovery in the interphase despite lower protein concentrations in the same phase. This could be due to the specific stabilizing agents in PAXgene tubes, which may promote DNA partitioning to the interphase by altering its interactions with plasma proteins or phase-forming components. Although the stabilization reagent is not disclosed, the manufacturer claims that the tube is free of cross-linking substances and does not chemically modify cfDNA [6]. Another notable observation was that pooled plasma from tube P had a reddish hue, possibly indicating hemolysis [46]. This was also reflected in the final ATPS, where the interphase appeared more red than yellow like in the other tubes. This suggests that cell lysis was not entirely prevented [42], with released hemoglobin likely aggregating in the interphase. This may explain the divergent DNA and protein measurements observed in the interphase of tube P, either due to interference with the analytical assays or altered distribution behavior.

Overall, the ATPS proved robustness across different tube types, achieving high DNA recovery in the bottom phase and a controlled distribution of plasma proteins. PAXgene tubes exhibited distinct behavior, warranting further investigation. For consistency, Streck or

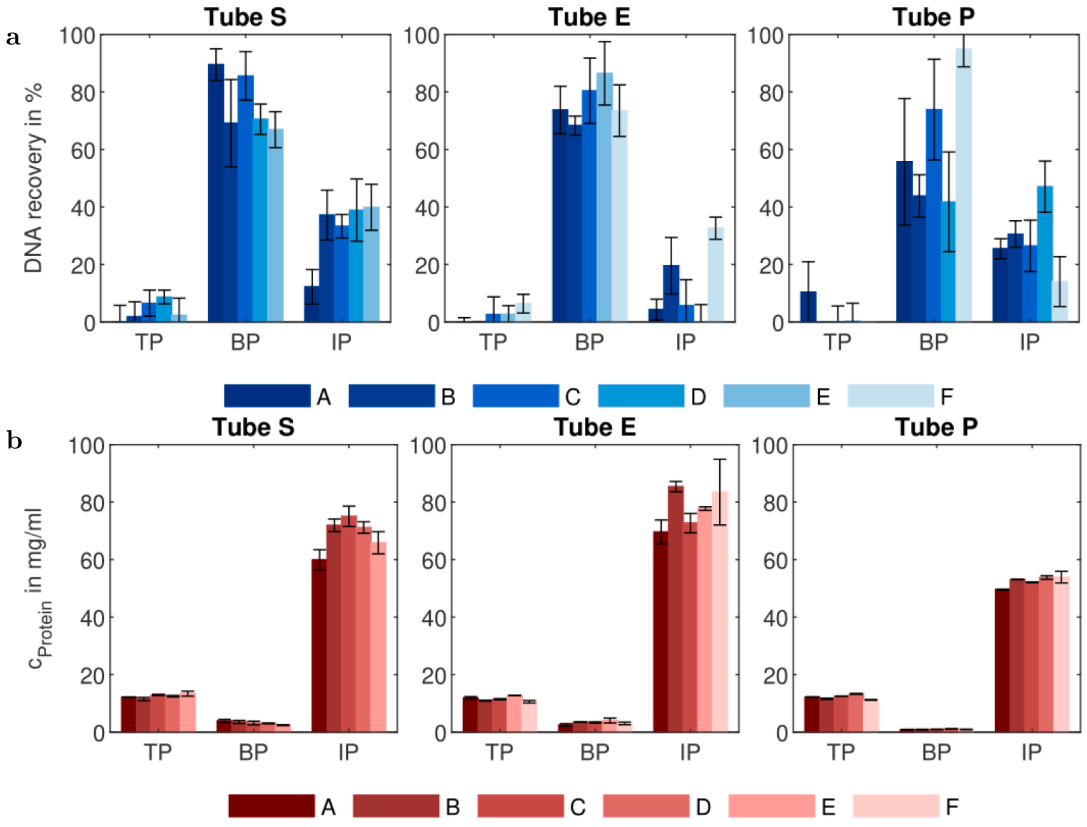

Fig. 5. (a) 160 bp DNA recovery and (b) plasma protein concentration in the top phase (TP), bottom phase (BP), and interphase (IP) of 19.5 % (w/w) PEG 1000/13.8 % (w/w) phosphate/37.7 % (w/w) plasma ATPS with plasma from individual donors (A-F) from EDTA (E), Streck (S) or PAXgene (P) blood collection tubes. Partitioning experiments were performed in duplicate and samples were measured in triplicate.

EDTA tubes may be preferred going forward.

3.2.2. Plasma variability

Another factor potentially affecting phase behavior and recovery outcomes in an ATPS composed of plasma is the plasma variability across individuals. Differences in plasma composition can arise due to variable water, protein, and metabolite contents resulting from donor-specific characteristics such as genetics, environment, lifestyle, and health status [47]. To investigate this, the same PEG 1000 system was examined using plasma from individual anonymous donors (A-F), each collected in at least two of the tube types S, E, or P. Fig. 5 displays the resulting DNA recovery and protein concentration across the top phase (TP), bottom phase (BP), and interphase (IP).

DNA recovery in the top phase was negligible across all plasmas and tube types. Most DNA partitioned into the bottom phase while the plasma protein bulk primarily accumulated at the interphase, consistent with pooled plasma experiments. This trend was evident across all plasmas tested for tube types S and E.

For tube S systems, DNA recovery in the bottom phase ranged from 67 % (donor E) to 90 % (donor A). Interphase recoveries were similar across plasmas, reaching up to 40 %, except for donor A, where it was only 12 %. This resulted in total recoveries exceeding 100 % for most plasmas except for donor A. This discrepancy may be due to interference from the stabilizing reagent, as it has been suggested that Streck tubes contain a formaldehyde-releasing substance that can chemically modify DNA through crosslinking [6], potentially leading to an overestimation of DNA quantities. This effect may be more pronounced in the interphase due to the high protein content, which could exacerbate interference with the fluorescence measurements. Warton et al. [43] reported contamination with high molecular weight DNA in Streck tubes, which may have accumulated in the interphase in this study. However, these explanations seem unlikely, as all samples were background-corrected using appropriate blank ATPS phase samples, and the effect was not observed consistently across all experiments with individual or pooled plasma. Another explanation is the trapping of bottom-phase liquid in the aggregated protein interphase, which could lead to an overestimation of the DNA content in the interphase.

The DNA and protein distribution in tube type E systems was similar to that of type S, with DNA recoveries ranging from 67 % (donor B) to 86 % (donor E). Interphase recoveries were lower overall, with some plasmas (donors A, C, and E) exhibiting values below the detection limit.

In contrast, tube P systems exhibited less reproducible results, with higher standard deviations and lower protein concentrations overall. DNA recovery in the bottom phase ranged from 42 % (donor D) to 95 % (donor F). Notably, the protein concentration in tube P systems was also lower in the bottom phase (around 1 mg/ml compared to 3 mg/ml for tubes S and E) and in the interphase (up to 53 mg/ml, compared to 69–85 mg/ml for tube E and 60–75 mg/ml for tube S). This suggests potential interference of either the stabilization reagent or partial hemolysis of the tube P samples, which showed a reddish hue, affecting protein absorbance readings [46].

Protein concentrations in the top phase were constant at approximately 12 mg/ml across all plasmas and tube types. In the bottom phase, concentrations plateaued at around 3 mg/ml for tubes S and E. This threshold effect is likely due to solubility limits in the PEG- and salt-rich phases [27] and could explain why the variations within the different plasmas were highest in the interphase, where all supplementary proteins excluded from the top and bottom phases tended to aggregate. This effect underscores a key advantage of the ATPS-based capture, as plasma composition variability does not significantly affect protein distribution in the bottom phase, ensuring reproducible outcomes.

Although some variability in DNA recovery was observed, particularly with tube type P samples, tubes S and E demonstrated consistent trends across donor samples. With plasma from these tubes, acceptable DNA recovery and low protein contamination in the bottom phase were achieved, highlighting the reproducibility and reliability of the ATPS-based capture step. Nevertheless, further optimization may be needed to address the variability introduced by specific tube formulations and improve performance with hemolyzed samples.

3.3. DNA concentration

3.3.1. ATPS formation with solid components

One of the major challenges in liquid biopsy is the inherently low concentration of cfDNA, especially during early-stage cancer detection when cfDNA levels are at their lowest [48]. To increase the likelihood of detecting rare cfDNA variants, a logical approach is to increase the plasma volume to be processed.

The plasma input volume can be increased without altering the relative concentrations of PEG and salt by adding the phase-forming components as solids directly into the plasma. This approach eliminates the need for pre-dissolving of the components, and the water content of the stock solutions is replaced with plasma [20]. This method also offers the advantage of preventing transiently high PEG and salt concentrations that could lead to DNA loss through precipitation [49], as the components gradually dissolve in plasma. Additionally, using solid components is more convenient for ready-to-use extraction kits, as the reduced water content decreases weight during logistics, reduces the risk of contamination, and extends shelf life. To assess the effectiveness of this strategy, DNA and protein recoveries were compared between ATPS prepared with pre-dissolved components (Fig. 5) and those prepared with solid phase-forming components (Fig. 6), using individual plasma samples (donors A-E) collected in Streck tubes.

By adding the components as solids, 29.0 % (w/w) of the water from the pre-dissolved stock solutions could be replaced with plasma without altering the amount of PEG and phosphate, resulting in 66.7 % (w/w) plasma in the ATPS. This approach not only increases the amount of extractable cfDNA but also minimizes the dilution associated with the addition of phase-forming components. DNA recoveries between the two preparation methods were comparable across all plasma samples, confirming consistent performance. The DNA recovery in the interphase was slightly lower when solid components were used, possibly due to reduced DNA precipitation attributed to the slower dissolution of PEG. The most notable difference was an increased protein concentration in the interphase when solid components were used (over 110 mg/ml compared to approximately 70 mg/ml). This was expected due to the higher plasma input volume and was visibly reflected in the thicker interphase. Despite this, phase formation and volume ratios were similar between preparation methods. Importantly, protein concentrations in the bottom phase remained unchanged despite the increased plasma volume, underscoring the ability of the ATPS capture step for controlled protein removal. This control could be particularly valuable for a subsequent lysis step, allowing precise adjustment of lysing agent quantities based on the known protein concentration in the bottom phase.

To evaluate the scalability of the solid-components plasma ATPS, the PEG 1000 system was prepared using 4 ml of pooled plasma. The phase volume ratio and the DNA and protein partitioning behavior remained consistent (Fig. S3). DNA recovery in the bottom phase was 80 %, with a protein concentration of 4.5 mg/ml.

These findings highlight the feasibility of dissolving solid phase-forming components directly into plasma. This method simplifies the preparation process, maximizes the cfDNA concentration available during extraction, and presents a scalable solution for processing larger plasma volumes.

3.3.2. Phase volume ratio manipulation

Another advantage of ATPS is the potential to concentrate target molecules by adjusting the phase volume ratio (V_R). Five systems were prepared along the same tie-line but with different V_R to investigate if the DNA could be concentrated in the bottom phase by reducing its volume without changing phase equilibrium composition [50]. To ensure comparability, all systems contained the same amount of plasma,

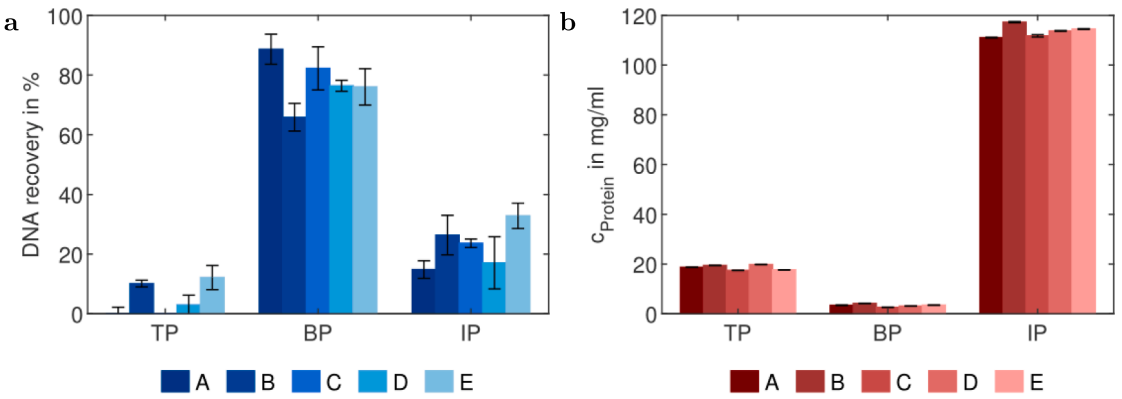

Fig. 6. (a) 160 bp DNA recovery and (b) plasma protein concentration in the top phase (TP), bottom phase (BP), and interphase (IP) of 19.5 % (w/w) PEG 1000/13.8 % (w/w) phosphate/66.7 % (w/w) plasma ATPS with solid components for different plasma donors (A-E) from Streck tubes. Partitioning experiments were performed in duplicate and samples were measured in triplicate.

with water added as needed to maintain the total volume. The exact compositions of the investigated system points are found in Table S3 in the supplementary material. In the first experiment, systems were spiked with 200 ng/ml of a 160 bp DNA fragment, while in the second experiment, a concentration of 100 ng/ml was used. DNA recovery and concentration, as well as protein concentration in the phases, are shown in Fig. 7.

A plateau in DNA concentration was observed in the bottom phase instead of the expected increase with decreasing phase volume. The plateau occurred at approximately 500 ng/ml for a 200 ng/ml input and at 200 ng/ml for a 100 ng/ml input across all tested V_R, except at V_R = 5.3, where the DNA concentration in the bottom phase was only half the plateau value. One hypothesis was that the plateau reflects the solubility limit of DNA in the bottom phase. However, this hypothesis is unlikely because the plateau was observed at 200 ng/ml when the input concentration was lowered to 100 ng/ml, even though up to 500 ng/ml DNA could theoretically partition into the bottom phase. Instead, the limiting factor appears to be the DNA concentration in the interphase, as comparable values were observed for both input concentrations. This data suggests that any DNA unable to accumulate in the interphase partitions into the bottom phase, explaining the observed plateau behavior.

Consequently, DNA recovery in the bottom phase and overall DNA recovery decreased with increasing V_R. The most likely explanation for the incomplete mass balances observed at $V_R \geq 3.1$ is the high amount of PEG 1000 required for phase formation, exceeding 27 % (w/w) for this specific tie-line. This elevated PEG concentration possibly caused irreversible DNA precipitation [51], contributing to the observed losses.

Protein distribution exhibited comparable trends across the two experiments, regardless of the DNA input amount. Protein concentration in the top phase plateaued at 10 mg/ml, while protein concentration in the bottom phase decreased as the bottom phase volume decreased. This result is counterintuitive, as one would expect both DNA and protein concentrations in the bottom phase to increase. Notably, protein concentration in the interphase peaked at V_R = 2.1 but decreased for V_R = 5.3, potentially due to the precipitating effects of the high PEG 1000 concentration [52]. This denaturation could prevent proper protein detection, mirroring the challenges observed with DNA, as total recoveries declined at higher V_R.

The same experiment was repeated using solid phase-forming components added directly to plasma to investigate whether the slower solubilization of PEG would improve DNA recovery. The plasma content in the solid component systems was higher than in the liquid component systems and was kept constant across all V_R values. All systems were spiked with 100 ng/ml DNA for comparability.

Like the liquid component systems, DNA recoveries in the bottom phase decreased with increasing VR when using solid components. However, bottom phase recoveries were approximately 19 % higher across all V_R, while interphase recoveries were lower, confirming that adding the components as solids reduces DNA precipitation. Despite this notable improvement, bottom phase recovery at V_R = 5.3 remained low (32 %), indicating that the PEG concentration was still too high to achieve an adequate DNA concentration. An additional experiment was conducted using a system point on a shorter tie-line, which required less PEG 1000 and salt, to improve DNA recovery and concentration while minimizing losses. While this adjustment reduced DNA precipitation, the lower PEG levels also decreased protein precipitation, shifting protein distribution from the interphase to the bottom phase (data not shown). These findings suggest that an optimal PEG concentration is essential to effectively precipitate plasma proteins while preventing DNA co-precipitation or entrapment, ensuring efficient DNA extraction and concentration.

3.4. Combined purification and concentration

Another approach to increase the DNA concentration involved removing liquid from the DNA-containing phase after the ATPS capture step. For this purpose, EchoLUTION purification plates with a custom matrix filling volume of 500 µl provided by BioEcho were used. The purification plates follow a reverse elution principle, where target DNA flows through while contaminants such as salt and residual proteins are retained [15]. A so-called conditioning step is required to remove the storage solution before the target solution is applied to the matrix. The standard protocol, according to the manufacturer's instructions, specifies a conditioning and elution centrifugation rate of 1000 x g for 1 min. In the following experiment, the conditioning and elution rates were modified to achieve simultaneous removal of contaminants and liquid, effectively concentrating the DNA in the flow-through. The flow-through volume, DNA concentration, and recovery for different combinations of conditioning and elution rates are presented in Fig. 8.

The flow-through volume decreased with decreasing elution rates, but increasing the conditioning speed achieved an even more significant reduction. At a conditioning rate of 3000 x g, a higher removal of storage solution effectively "dried out" the matrix, enabling it to retain more input liquid within the pores and between particles [15]. Combined with a lower elution rate, which increases the residence time of the liquid in the matrix, an efficient volume reduction of the flow-through was achieved. By combining conditioning at 3000 x g with elution at 750 x g, the input volume was reduced by 35 %, while DNA concentration was enhanced by 12 % compared to the standard protocol.

Interestingly, DNA recovery was not significantly affected by the increased conditioning rate. However, lower elution centrifugation rates led to a decrease in DNA recovery. This could be attributed to insufficient centrifugal force to overcome capillary forces and prolonged residence time within the matrix, which may cause DNA retention

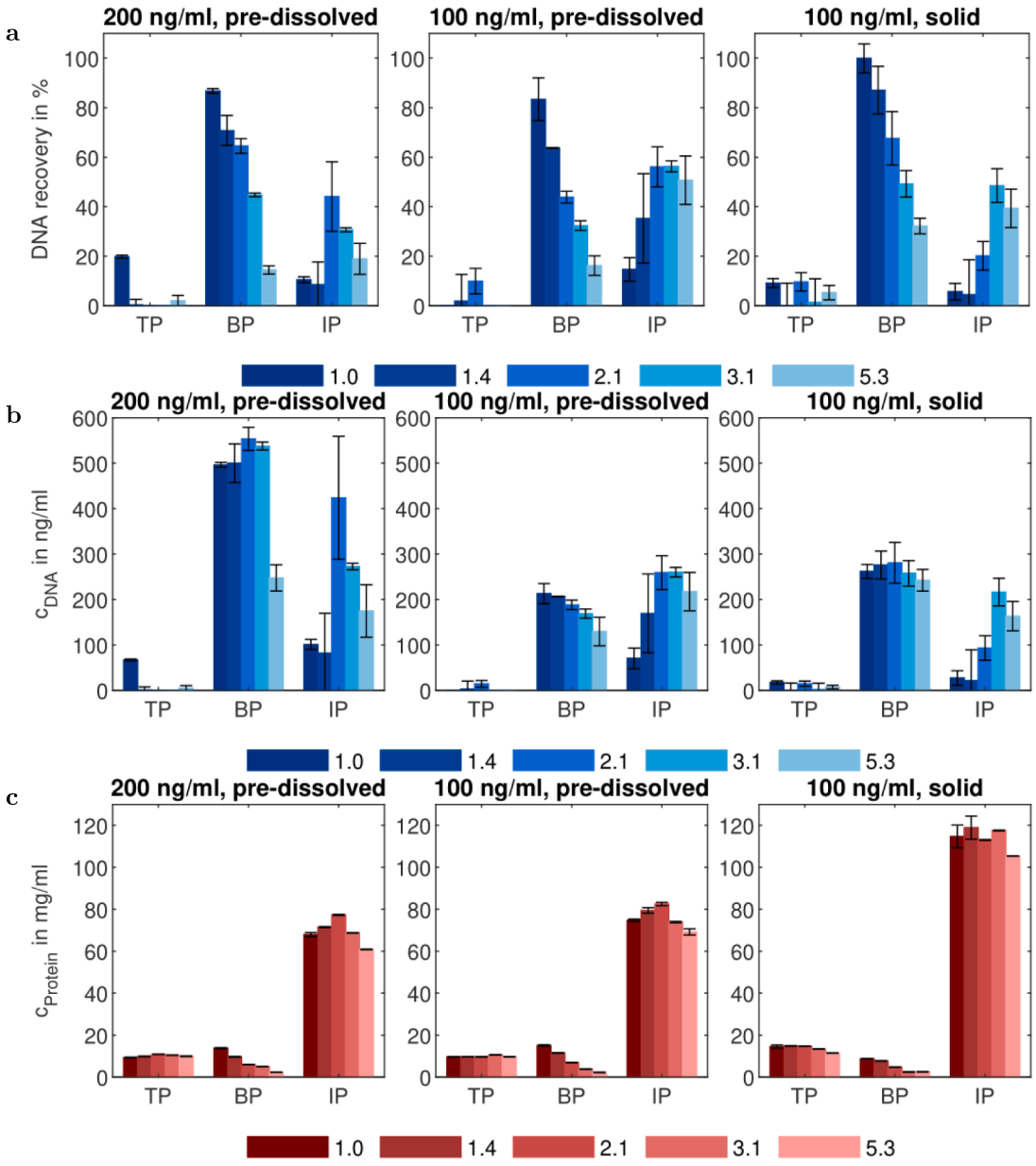

Fig. 7. (a) 160 bp DNA recovery, (b) concentration, and (c) plasma protein concentration in the top phase (TP), bottom phase (BP), and interphase (IP) of five PEG 1000/phosphate/plasma ATPS with phase volume ratios ranging from 1.0 to 5.3. ATPS were spiked with 200 ng/ml or 100 ng/ml DNA and were prepared with pre-dissolved or solid components. Partitioning experiments were performed in duplicate and samples were measured in triplicate.

between particles or diffusion into pores [53]. Supplementary experiments demonstrated that unspecific adsorption to plate walls and frit material accounted for no more than 5 % of DNA loss (data not shown). These findings suggest that the remaining DNA loss likely stems from electrostatic or hydrophobic interactions with the matrix or entrapment within its structure [54].

The desalting capacity of the purification plate was evaluated under the adapted conditions (conditioning 3000 x g, elution 750 x g). Conductivity measurements with increasing phosphate stock concentrations (Fig. 9) demonstrated that salt removal remained as effective as the standard protocol for up to 30 % (w/w) input salt. Less than 5 % of the salt remained in the flow-through, corresponding to conductivity values below 2 mS/cm. Additionally, purification experiments of blank bottom phases revealed a reduction in plasma protein concentrations from 4 mg/ml to below 0.5 mg/ml, with successful protein removal confirmed via SDS-PAGE (Fig. S4). However, when plasma was purified without a prior ATPS capture step, only 37.5 % of total protein was removed, leaving a substantial amount of protein exceeding 30 mg/ml. These results highlight the necessity of the ATPS capture step for significant protein content reduction, as the plasma protein concentration is too high for the purification plates alone. Conversely, the purification step is essential for removing residual protein and reducing the high salt concentration introduced by ATPS, which can inhibit downstream applications such as PCR. Indeed, preliminary quantitative PCR runs confirmed that amplification was only possible for desalted samples (data not shown). While some DNA loss remains inevitable, the modified reverse elution protocol demonstrates strong potential for achieving combined DNA purification and concentration, towards meeting the requirements for amplification-based downstream applications.

4. Conclusions

This study demonstrates the potential of ATPS as a robust and efficient approach for cfDNA capture from plasma. The presented PEG/

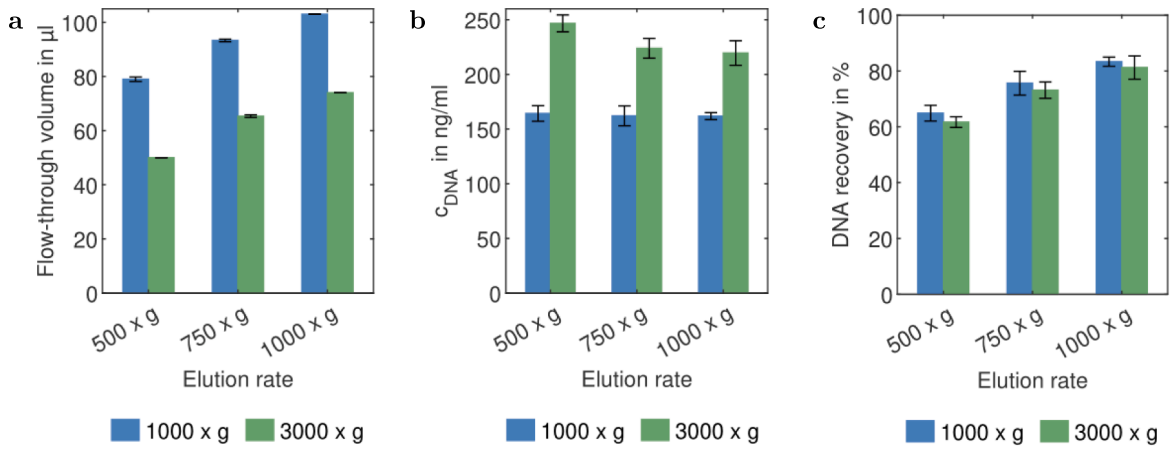

Fig. 8. (a) Flow-through volume, (b) concentration, and (c) recovery of a 160 bp DNA fragment after reverse elution. An input volume of 100 µl of 200 ng/ml DNA in water was applied per 500 µl matrix-filled well in triplicate. Conditioning centrifugation rates of 1000 x g and 3000 x g were tested in combination with elution rates of 500 x g, 750 x g, and 1000 x g.

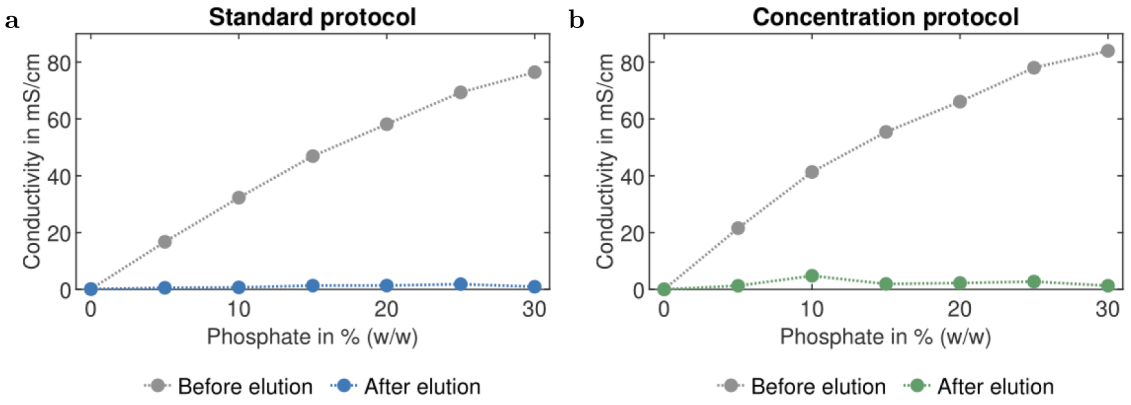

Fig. 9. Conductivity measurements before and after reverse elution using: (a) standard protocol (conditioning and elution at 1000 x g), (b) concentration protocol (conditioning at 3000 x g, elution at 750 x g). Increasing amounts of phosphate salt were used as input (100 µl per well filled with 500 µl matrix).

phosphate systems achieved DNA purification and concentration by selectively partitioning DNA into the bottom phase while effectively separating it from the plasma protein bulk. Despite variability in plasma and blood collection tube types, consistent DNA recovery and controlled protein removal were observed, underscoring the robustness of the ATPS capture step. The addition of solid phase-forming components enabled increased plasma input without compromising DNA recovery and protein distribution, highlighting this method's scalability and practicality. Additionally, an adapted reverse elution protocol demonstrated promise as a tool for streamlined downstream processing of the DNA-containing, salt-rich solution after ATPS-based capture. Nonetheless, further improvements are necessary to remove residual proteins and phase-forming components that could interfere with downstream analyses such as PCR or NGS. Ongoing efforts focus on implementing a lysis step post-capture, which, when combined with reverse elution, could streamline the simultaneous removal of salts and proteins without relying on adsorption-based processes. These findings establish a solid foundation for developing a scalable and reliable ATPS-based workflow for efficient cfDNA extraction in liquid biopsy applications.

CRediT authorship contribution statement

Rafaela Meutelet: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Lea J. Bisch:** Validation, Investigation, Writing – review & editing. **Benedikt C. Buerfent:** Conceptualization, Resources, Writing – review & editing, Supervision. **Johannes Oldenburg:** Resources, Writing – review & editing, Supervision. **Timo Hess:** Conceptualization, Resources, Writing – review & editing. **Heiko Rühl:** Resources, Writing – review & editing. **Jürgen Hubbuch:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Generative AI and AI-assisted technologies in the writing process

While preparing this work, the authors used ChatGPT (OpenAI, San Francisco, CA, USA) to improve the readability and language of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Declaration of competing interest

Benedikt Buerfent and Timo Hess declare employment by BioEcho Life Sciences GmbH. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2025.132884.

Data availability

Data will be made available on request.

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